

Attenuation of Colon Inflammation through Activators of the Retinoid X Receptor (RXR)/Peroxisome Proliferator-activated Receptor γ (PPAR γ) Heterodimer: A Basis for New Therapeutic Strategies

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Abstract

The peroxisome proliferator-activated receptor γ (PPAR γ) is highly expressed in the colon mucosa and its activation has been reported to protect against colitis. We studied the involvement of PPAR γ and its heterodimeric partner, the retinoid X receptor (RXR) in intestinal inflammatory responses. PPAR $\gamma^{+/-}$ and RXR $\alpha^{+/-}$ mice both displayed a significantly enhanced susceptibility to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis compared with their wild-type littermates. A role for the RXR/PPAR γ heterodimer in the protection against colon inflammation was explored by the use of selective RXR and PPAR γ agonists. TNBS-induced colitis was significantly reduced by the administration of both PPAR γ and RXR agonists. This beneficial effect was reflected by increased survival rates, an improvement of macroscopic and histologic scores, a decrease in tumor necrosis factor α and interleukin 1 β mRNA levels, a diminished myeloperoxidase concentration, and reduction of nuclear factor κ B DNA binding activity, c-Jun NH₂-terminal kinase, and p38 activities in the colon. When coadministered, a significant synergistic effect of PPAR γ and RXR ligands was observed. In combination, these data demonstrate that activation of the RXR/PPAR γ heterodimer protects against colon inflammation and suggest that combination therapy with both RXR and PPAR γ ligands might hold promise in the clinic due to their synergistic effects.

Key words: colitis • inflammatory bowel disease • nuclear receptors • tumor necrosis factor α • signal transduction pathway

Introduction

The peroxisome proliferator-activated receptor γ (PPAR γ)¹ is a nuclear receptor that controls the expression of several genes involved in metabolic control. PPAR γ is activated by fatty acid derivatives, thiazolidinediones, and

certain nonsteroidal antiinflammatory drugs (1–3). Thiazolidinedione PPAR γ agonists, such as troglitazone, rosiglitazone, and pioglitazone, have been or are currently being used as insulin-sensitizing drugs in the treatment of the type 2 diabetes (4). In addition to these effects on glucose

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¹Abbreviations used in this paper: IBD, inflammatory bowel disease; JNK, c-jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase;

MPO, myeloperoxidase; NF, nuclear factor; PPAR γ , peroxisome proliferator-activated receptor γ ; RXR, retinoid X receptor; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

homeostasis, *in vitro* studies have shown that PPAR γ activators could have antiinflammatory effects. PPAR γ activators were able to limit the production of inflammatory mediators such as inflammatory cytokines produced by human activated monocytes-macrophages and intestinal epithelial cells through an inhibition of transcription driven by nuclear factor (NF)- κ B and activating protein 1 (AP-1) transcription factors (5–7). Recently, PPAR γ agonists have been reported to attenuate colitis in a murine model in which inflammation was induced by administration of dextran sodium sulfate (7). This observation suggested that PPAR γ activators may be useful in the treatment of patients with inflammatory bowel disease (IBD). IBD encompasses several chronic diseases, which are characterized at least in part by an overproduction of pathogenic inflammatory cytokines such as TNF- α and IL-1 β , leading to the activation of the NF- κ B and c-Jun NH $_2$ -terminal kinase (JNK)/p38 mitogen-activated protein kinase (MAPK) pathways (8–10).

To activate transcription, PPAR γ requires heterodimerization with the retinoid X receptor (RXR). The RXR/PPAR γ heterodimers are permissive to activation by both PPAR γ and RXR ligands. Therefore, several of the biological effects of PPAR γ activation, such as insulin sensitization, can be reproduced by specific RXR agonists or rexinoids. Proof of a role for the RXR/PPAR γ heterodimer in intestinal inflammation has been limited to pharmacological studies demonstrating the efficacy of PPAR γ agonists. In this study, we investigated the potential effects of both PPAR γ and RXR in an experimental animal model in which colitis was induced by intrarectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS). We provide genetic evidence of an involvement of both PPAR γ and RXR in colon inflammation, by showing that PPAR $\gamma^{+/-}$ and RXR $\alpha^{+/-}$ mice were significantly more susceptible to inflammation than their wild-type littermates. Furthermore, we confirm the protective effects of PPAR γ ligands in a different experimental colitis model than the one used by Su et al. (7). Most importantly, we demonstrate for the first time that RXR agonists were equally effective as PPAR γ agonists in reducing intestinal

inflammation. In addition, rexinoids have a marked synergistic effects with PPAR γ agonists on inflammation. In combination, our data emphasize the importance of both partners of the RXR/PPAR γ heterodimer in the regulation of colon inflammation. The synergistic antiinflammatory activities of RXR and PPAR γ agonists suggests that coadministration of low doses of PPAR γ and RXR agonists might be worth exploring in human IBD.

Materials and Methods

Materials

The PPAR γ and RXR agonists were synthesized at Ligand Pharmaceuticals. TNBS was purchased at Fluka.

Induction of Colitis and Study Design

Animal experiments were performed in accredited establishments (N $^{\circ}$ B59-108 and B67-218-5) according to governmental guidelines N $^{\circ}$ 86/609/CEE. Animals were group housed (6–8/cage) and had free access to regular rodent chow and tap water. For induction of colitis, mice, which were anesthetized for 90–120 min, received by intrarectal administration 40 μ l of a solution of TNBS (150 mg/kg) dissolved in 0.9% NaCl and mixed with an equal volume of ethanol (50% ethanol). Control mice received 50% ethanol or a saline solution using the same technique. Animals were killed by cervical dislocation 2 or 5 d after TNBS administration. First, wild-type male Balb/c mice were used in the intervention studies with the PPAR γ and RXR agonists, rosiglitazone (5–50 mg/kg/day), troglitazone (50–200 mg/kg/day), or LG101305 (5–50 mg/kg/day). These compounds were administered once daily by oral gavage (11), starting either 2 d before (preventive mode) or just after the induction of colitis (therapeutic mode). A general outline of the different intervention studies is represented in Fig. 1. In a second set of experiments, PPAR $\gamma^{+/-}$ and RXR $\alpha^{+/-}$ mice or their respective wild-type littermates (both on a 129/Sv background) were used. Heterozygote mice were used because homozygous PPAR $\gamma^{-/-}$ and RXR $\alpha^{-/-}$ mice are embryonic lethal. These 129/Sv mice were killed 2 d after the induction of colitis.

Macroscopic and Histologic Assessment of Colitis

The colon was examined under a dissecting microscope ($\times 5$) to evaluate the macroscopic lesions according to the Wallace cri-

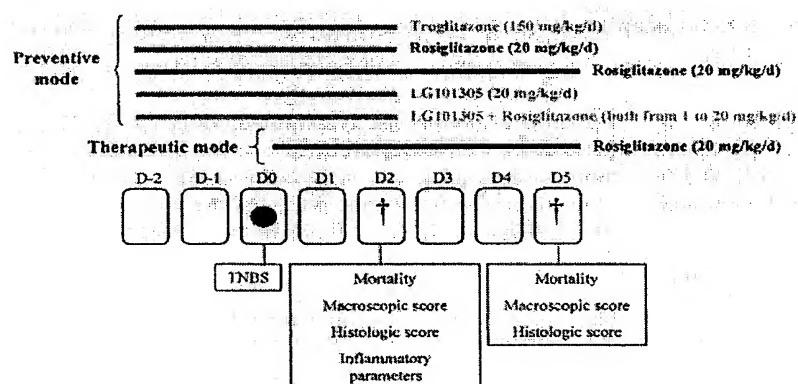


Figure 1. Design of the animal intervention studies. TNBS (black ellipse) was administered intrarectally on day (D) 0 and mice were killed 2 or 5 d later. The rexinoid (LG101305), the PPAR γ agonists, such as rosiglitazone and troglitazone, or the combination of a rexinoid and a PPAR γ agonist were administered at the indicated doses 2 d before the induction of colitis (preventive mode). The therapeutic effects were evaluated in mice receiving the same dose of rosiglitazone just after TNBS administration (therapeutic mode). Treatment with PPAR γ and/or RXR agonists was evaluated at day 2 or 5 by scoring for mortality, determination of macroscopic and histologic inflammation scores, and measurements of inflammatory parameters (MPO levels, TNF- α and IL-1 β mRNA, NF- κ B pathway, and MAPK activity).

Table 1. Mouse Oligonucleotide Sequences

Primer	Sequence
TNF- α AS	5'-GGG-AGT-AGA-CAA-GGT-ACA-AC-3'
TNF- α S	5'-TCT-CAT-CAG-TTC-TAT-GGC-CC-3'
IL-1 β AS	5'-AGA-AGG-TGC-TCA-TGT-CCT-CAT-3'
IL-1 β S	5'-TTG-ACG-GAC-CCC-AAA-AGA-TG-3'

AS, antisense; S, sense.

teria. The Wallace score rates macroscopic colon lesions on a scale from 0 to 10 based on criteria reflecting inflammation, such as hyperemia, thickening of the bowel, and the extent of ulceration (12). A colon specimen located precisely 2 cm above the anal canal was cut into four parts. One part was fixed overnight in 4% paraformaldehyde acid and embedded in paraffin. Sections stained with hematoxylin and eosin were examined blindly by a pathologist and scored according to the Ameho criteria (13). This grading on a scale from 0 to 6 takes into account the degree of the inflammatory infiltrate, the presence of erosion, ulceration or necrosis, and the depth and surface extension of the lesions (13). The other parts of the colon were used for quantification of TNF- α and IL-1 β mRNA, myeloperoxidase (MPO), and NF- κ B DNA binding or MAPK activities.

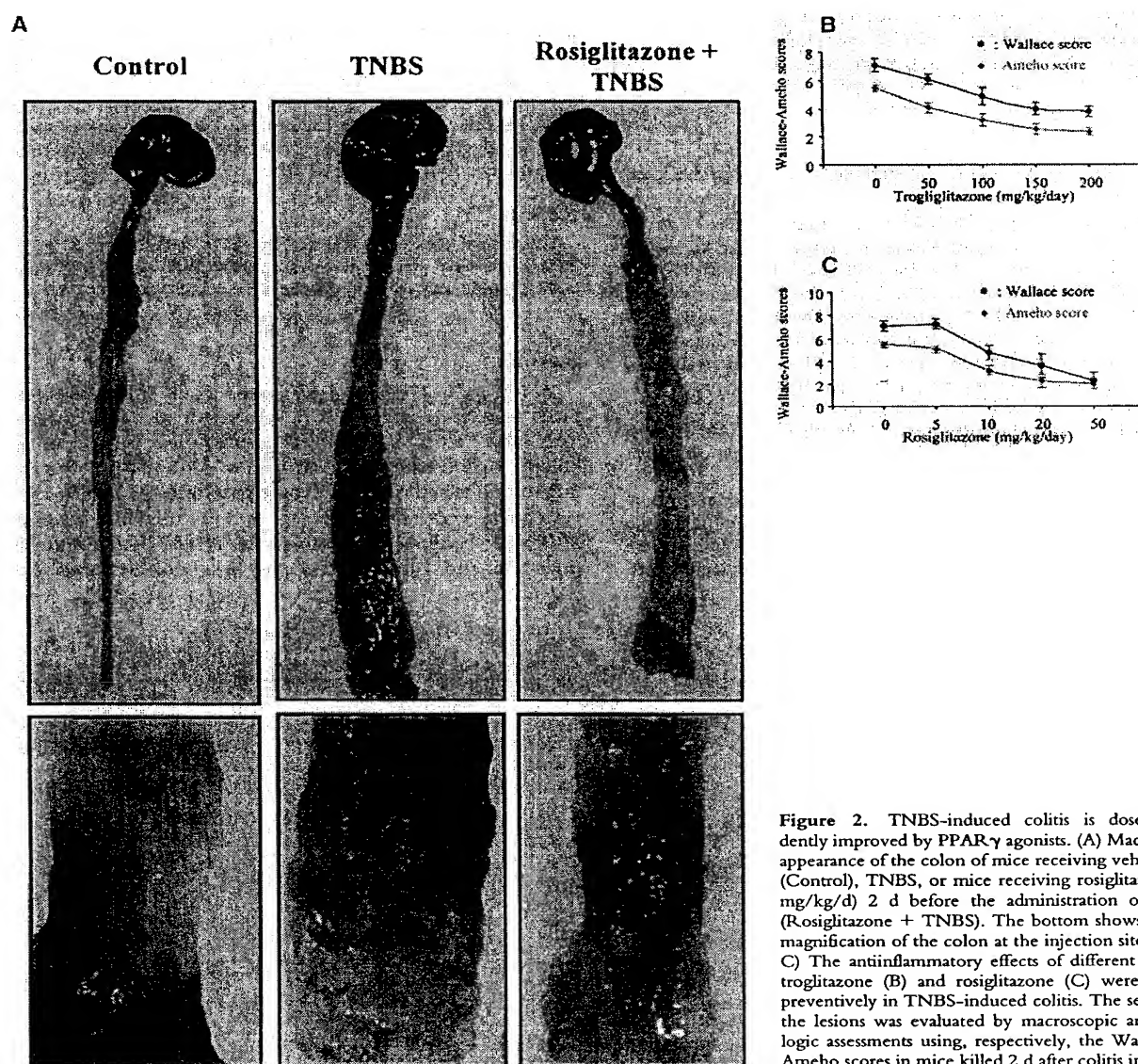


Figure 2. TNBS-induced colitis is dose-dependently improved by PPAR γ agonists. (A) Macroscopic appearance of the colon of mice receiving vehicle only (Control), TNBS, or mice receiving rosiglitazone (20 mg/kg/d) 2 d before the administration of TNBS (Rosiglitazone + TNBS). The bottom shows a larger magnification of the colon at the injection site. (B and C) The antiinflammatory effects of different doses of troglitazone (B) and rosiglitazone (C) were assessed preventively in TNBS-induced colitis. The severity of the lesions was evaluated by macroscopic and histologic assessments using, respectively, the Wallace and Ameho scores in mice killed 2 d after colitis induction.

Quantification of Protein and mRNA Expression in the Colon

Protein preparation and immunoblotting were performed as described (14). Total protein extracts were obtained by homogenization of tissues in an extraction buffer consisting of PBS with 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and a classical protease inhibitor cocktail. Total proteins were then separated by PAGE and electroblotted (14). Immunodetection with a secondary peroxidase-conjugated antibody and chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham Pharmacia Biotech). RNA was isolated from colon samples with the TRIzol reagent as described (15). After treatment at 37°C for 30 min with 20–50 units of DNase I RNase-free (Boehringer), total RNA (5–10 µg) was reverse transcribed into cDNA. The reverse transcription reaction mixture was amplified by PCR using sense and antisense primers specific for TNF-α and IL-1β (Table I; references 15 and 16). The samples were subjected to 40 PCR cycles (PerkinElmer). Quantification of cytokine cDNA was performed by electrophoresis in 2–3% agarose gel using an image analyzer (Gel Analyst; Clara Vision; reference 17). Results of cytokine measurements were expressed in proportion to the quantity of total RNA, in the same sample.

Quantification of NF-κB DNA Binding, JNK, and p38 Kinase Activities in the Colon

NF-κB Electromobility Shift Assay. Cellular protein extracts were prepared by homogenizing the colon in the following buffer: 20 mM Hepes, pH 7.9, 350 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM EGTA, 20% glycerol, 1% NP-40, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 2 µg/ml aprotinin, and 10 µM leupeptin. In vitro binding reactions of NF-κB in a total volume of 25 µl were initiated by incubation of 5 µg of nuclear protein extracts in a binding buffer containing 10 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl₂, 5% glycerol, 2 µg poly(dI-dC), and 0.4 mg/ml salmon sperm DNA (18). After 10 min of preincubation on ice, 50,000–100,000 cpm of ³²P end-labeled NF-κB oligonucleotide probe (5'-GATCCAAGGGACTTTCCATG-3' corresponding to the NF-κB binding sequence of the human IL-2 promoter) were added and the binding reaction was allowed to proceed for 20

min at 21°C. Then, DNA-protein complexes were resolved by electrophoresis on Tris-acetic acid-EDTA (TAE)-4% polyacrylamide gels at 4°C, and analyzed by autoradiography. As control, a 50-fold molar excess of cold NF-κB competitor oligonucleotide was added during preincubation.

JNK or p38 Kinase Assay. Colon was homogenized in TLB (triton lysis buffer): 20 mM Tris, pH 7.5, 137 mM NaCl, 2 mM EDTA, pH 7.5, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM NaPPi, 10% glycerol, 1 mM PMSF, and 10 µg/ml leupeptin. Soluble extracts were prepared by centrifugation at 12,000 rpm for 30 min at 4°C. The extracts (300 µg of protein) were incubated with 2 µl of anti-JNK or anti-p38 rabbit serum prebound to protein A-Sepharose (19). After 1 h of incubation at 4°C, the immunoprecipitates were washed twice with TLB and twice with kinase buffer: 25 mM Hepes, pH 7.5, 25 mM MgCl₂, 25 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), and 0.1 mM Na orthovanadate. Immune complex kinase assays were performed at 30°C for 20 min using 2 µg of glutathione-S-transferase (GST)-activating transcription factor (ATF)2(1-109), 50 µM ATP, and 3–10 µCi of [γ-³²P]ATP as substrates in 20 µl of kinase buffer (20). The reactions were terminated with Laemmli sample buffer, the products were resolved by SDS-PAGE (12%), and quantified after autoradiographic analysis as described (20).

Statistics

All comparisons were analyzed by the nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) test. Differences were judged statistically significant if the *P* value was <0.05.

Results

TNBS-induced Colitis Is Improved by PPARγ Agonists. First, we characterized the development of colitis in animals subjected to TNBS injection. Whereas control mice, killed 2 or 5 d after administration of 50% ethanol or a saline solution, had no macroscopic lesions in the colon, a severe colitis was induced as early as 2 d after administration of TNBS, resulting in death in 22 ± 6% of the animals (Fig. 2 A, and Table II). 5 d after induction of colitis, the

Table II. Preventive Administration of Rosiglitazone or Troglitazone Have Similar Therapeutic Effects on Colitis 2 d after TNBS Administration

	Control (n = 16)	TNBS (n = 23)	Rosiglitazone (n = 14) on TNBS-induced colitis	Troglitazone (n = 8) on TNBS-induced colitis
Mortality	0%	22 ± 6%	12.5 ± 12.5%	10.2 ± 8%
Wallace score	0 ± 0	7.14 ± 0.86*	3.6 ± 2‡	3.8 ± 1.5§
Ameho score	0 ± 0	5.5 ± 0.5*	2.3 ± 1.2‡	2.5 ± 1.5§
MPO values	2.5 ± 3.6	34 ± 16*	14 ± 20‡	18 ± 14§
TNF-α mRNA	0.84 ± 0.81	3.91 ± 3.94*	0.58 ± 0.69‡	0.45 ± 0.74§
IL-1β mRNA	0.32 ± 0.26	28.5 ± 31.1*	4 ± 3‡	4 ± 4§

*Denotes a *P* < 0.001 between control and TNBS-induced colitis.

‡Denotes a *P* < 0.01 between TNBS-induced colitis and mice with colitis treated with rosiglitazone.

§Denotes a *P* < 0.01 between TNBS-induced colitis and mice with colitis treated with troglitazone.

¶Denotes a *P* = 0.001 between TNBS-induced colitis and mice with colitis treated with rosiglitazone.

lesions were more severe with necrosis of the colon leading to mortality in $68 \pm 4\%$ of the animals. On a histologic level, no abnormalities were detected in control mice (Fig. 3 A). In sharp contrast, 2 d after the administration of TNBS, colon histology was characterized by large areas of ulceration with a neutrophilic infiltrate, necrosis extending deeply into the muscular layer (Fig. 3, B and C), and enhancement of MPO levels, a marker of neutrophil content (21; Fig. 4). Necrosis of the colon in mice surviving 5 d after TNBS administration involved $\sim 90\%$ of the specimen, and was so severe that it precluded evaluation of other parameters such as MPO, TNF- α , and IL-1 β mRNA levels, and activity of NF- κ B.

We then evaluated the effects of PPAR γ activation on TNBS-induced colon lesions by performing a detailed dose-response study, using two different synthetic PPAR γ agonists, i.e., troglitazone (Fig. 2 B; from 50 to 200 mg/kg/day) and rosiglitazone (Fig. 2 C; from 5 to 50 mg/kg/day). In the first experiments, PPAR γ agonists were used in a preventive mode and administered 2 d before colon lesions were induced. When animals were analyzed 2 d after the induction of colitis, both PPAR γ agonists significantly reduced mortality compared with untreated mice with colitis and improved macroscopic and microscopic aspects of the TNBS-induced colon lesions (Fig. 2 and Table II). Optimal effects were obtained with a dose of 150–200 mg/kg/d of troglita-

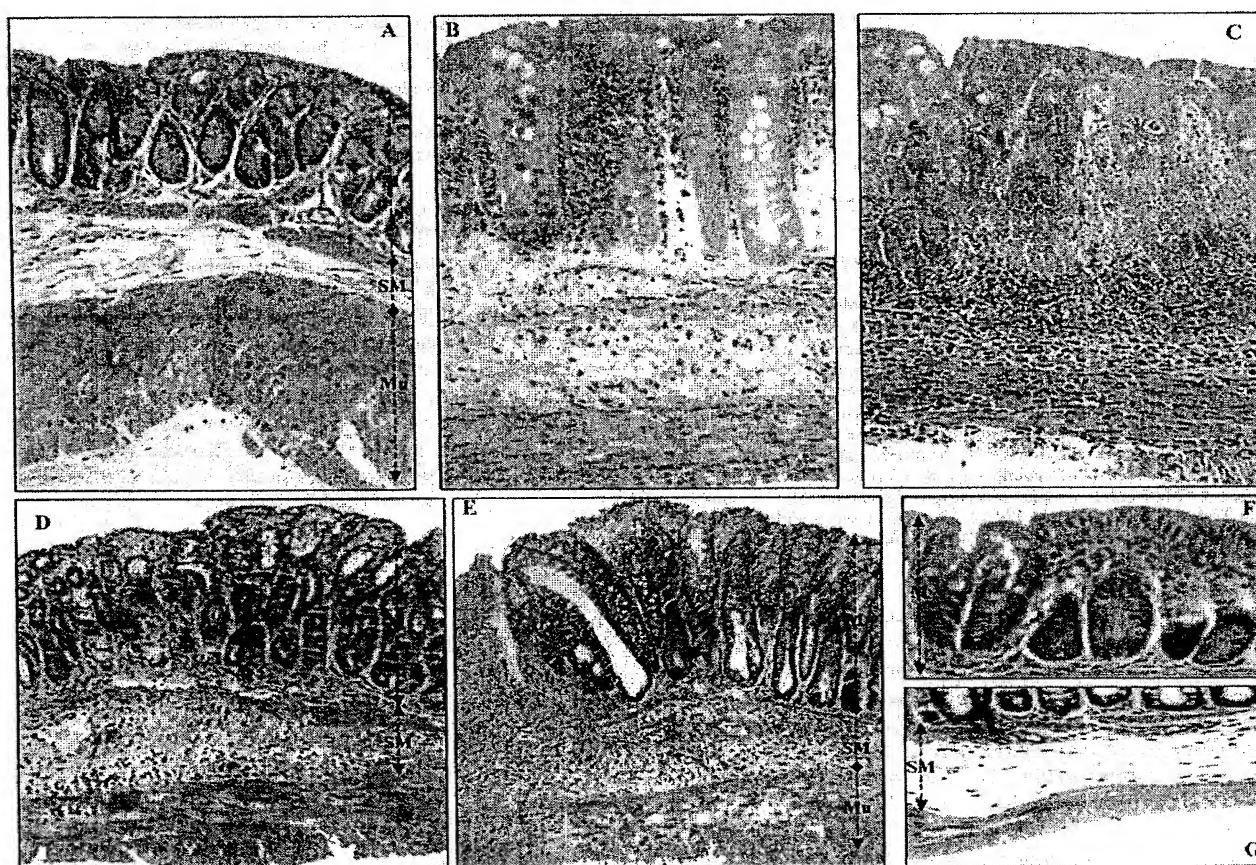


Figure 3. Representative histological sections of colon tissues of Balb/c mice. (A) Normal transperietal colon section of a vehicle-treated mouse with an Ameho score of 0 ($\times 250$). The different layers are indicated: M, mucosa; SM, submucosa; Mu, muscular layer. (B) Transperietal colon section (Ameho score 6) 2 d after the induction of colitis by TNBS. Thickening of the colon wall, with a predominant inflammatory infiltrate in the lamina propria, and necrosis extending deeply into the muscular and serosal layers are evident ($\times 400$). (C) Transperietal colon section (Ameho score 6) 5 d after the induction of colitis by TNBS. Parietal necrosis extending deeply into the muscular layer with the disappearance of cells in the mucosa is visible ($\times 250$). (D) Transperietal colon section of a mouse, which received rosiglitazone before TNBS administration. The mouse was killed 2 d after colitis induction. The Ameho score was graded 2. The picture shows a subepithelial edema with a diastasis of the crypts and a moderate inflammatory infiltrate in the mucosa and submucosa ($\times 250$). (E) Transperietal colonic section of mice treated with rosiglitazone after administration of TNBS. The pictures show mice killed 5 d after colitis induction. Ulceration extending into the submucosa, associated with a mucosal, submucosal, and muscular inflammatory infiltrate involving $<50\%$ of the specimen, is visible ($\times 250$). (F and G) Colon sections of mice that received rosiglitazone before TNBS administration. The mice were killed 2 d after colitis induction. In some cases, a total repair of the mucosa was observed (F; $\times 600$) despite the persistence of an in-depth focal necrosis in the submucosal layer (G; $\times 400$).

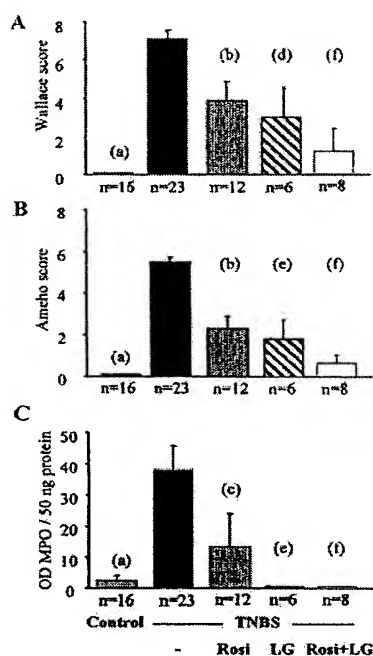


Figure 4. Effect of PPAR γ and RXR agonists on TNBS-induced colitis. Wallace macroscopic inflammation score (A), Ameho histologic score (B), and colon MPO levels (C) of mice receiving vehicle only (Control), TNBS, rosiglitazone (Rosi at 20 mg/kg/d), LG101305 (LG at 20 mg/kg/d), or rosiglitazone and LG101305 simultaneously (Rosi + LG both at 20 mg/kg/d) 2 d before the administration of TNBS. The number of mice is indicated and results are expressed as the mean \pm SEM. Animals were killed 2 d after TNBS treatment. (a) $P < 0.001$ in control mice vs. untreated TNBS colitis; (b) $P < 0.001$ and (c) $P = 0.009$ in untreated TNBS colitis vs. mice receiving rosiglitazone; (d) $P = 0.002$ and (e) $P < 0.001$ in untreated TNBS colitis vs. mice receiving LG101305; and (f) $P < 0.001$ in untreated TNBS colitis vs. mice receiving both rosiglitazone and LG101305.

zone and 20–50 mg/kg/d of rosiglitazone (Fig. 2, B and C). Consistent with their efficacy and potency as PPAR γ agonists in transfection assays, rosiglitazone was both a more potent and more efficacious antiinflammatory compound than troglitazone. These optimal doses were used in all following experiments. 5 d after induction of colitis, a significant decrease in both mortality and macroscopic lesion score was observed in mice that had received rosiglitazone preventively compared with untreated mice with colitis (Table III).

Parallel to the gross macroscopic inflammation, histological analysis also revealed major differences between TNBS animals and animals receiving PPAR γ agonists (Fig. 3, B and D). This was reflected by a significant decrease of the Ameho inflammation score both at 2 (Fig. 4 B) and 5 d (Table III) after TNBS administration. Administration of PPAR γ agonists dramatically reduced the inflammatory lesions, which consisted of smaller polymorphic inflammatory infiltrates, with mononuclear predominance, limited edema, and small focal necrotic lesions in close contact to regions with reepithelialization of the mucosal layer (Fig. 3 D). This improvement by the preventive administration of rosiglitazone

Table III. Rosiglitazone Used in a Preventive or Therapeutic Modes Improved Colitis 5 d after TNBS Administration

	Control (n = 16)	TNBS (n = 14)	Rosiglitazone on TNBS-induced colitis	
			Preventive mode (n = 16)	Therapeutic mode (n = 16)
Mortality	0 \pm 0%	68 \pm 4%*	19 \pm 9%†	14 \pm 8%‡
Wallace score	0 \pm 0	8.9 \pm 1.4*	3.5 \pm 0.6‡	5.8 \pm 0.9§
Ameho score	0 \pm 0	6 \pm 0*	2.1 \pm 1.1‡	4.1 \pm 0.7

* $P < 0.0001$ between control and TNBS-induced colitis.

† $P < 0.0001$ between TNBS-induced colitis and mice with colitis treated with rosiglitazone.

§ $P = 0.02$ between TNBS-induced colitis and mice with colitis treated with rosiglitazone.

|| $P = 0.001$ between TNBS-induced colitis and mice with colitis treated with rosiglitazone.

was associated with a significant decrease of MPO levels 2 d after TNBS administration (Fig. 4 C). Interestingly, in certain cases, total repair of the mucosal and muscularis mucosa layers was observed despite the persistence of some in-depth lesions (Fig. 3 F). These lesions were either in the form of a mononuclear infiltrate in the submucosa and the muscular layers, or in some cases in the form of focal necrosis of the ischemic type in the muscular layer (Fig. 3 G).

In the experiments described above, administration of PPAR γ agonists started before TNBS was administered. Therefore, we next analyzed whether the administration of

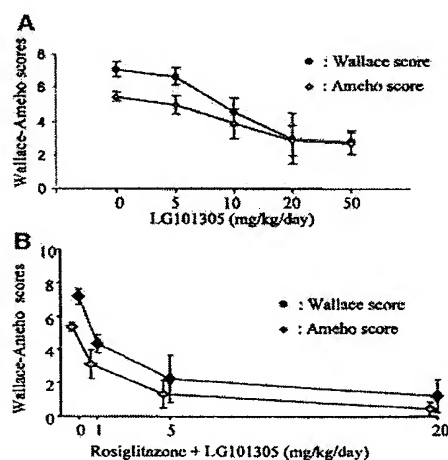


Figure 5. PPAR γ -RXR agonists have a synergistic effect on colitis. The antiinflammatory effects of different doses of the RXR agonist LG101305 (A) and of the simultaneous administrations of rosiglitazone (from 1 to 20 mg/kg/d) and LG101305 (from 1 to 20 mg/kg/d) (B) were assessed in TNBS-induced colitis. The severity of the lesions was evaluated by macroscopic and histologic assessments using, respectively, the Wallace and Ameho scores in mice killed 2 d after colitis induction.

rosiglitazone immediately after the induction of colitis was also effective in reducing lesion intensity. In this therapeutic mode, rosiglitazone also significantly improved the macroscopic lesions and mortality compared with untreated mice with colitis (Table III). This macroscopic improvement was again associated with a significant decrease of the histologic score (Table III and Fig. 3 E). These data prove that PPAR γ agonists can not only prevent lesion development, but are also effective in reducing established inflammatory lesions in the colon.

RXR Agonist Synergizes with PPAR γ Agonists in Improving Colitis. As PPAR γ forms a "permissive" heterodimer with RXR, we next used the selective and potent RXR

agonist LG101305 to analyze whether activation of RXR could mimic any of the beneficial effects of PPAR γ activation on TNBS-induced colon lesions. In dose-response studies, LG101305 at a dose of 50 mg/kg/d achieved a maximal effect similar to that as observed with the maximally efficacious dose of rosiglitazone (Figs. 2 C and 5 A). This improvement was associated with a significant decrease in the histologic lesion score and a normalization of MPO levels (Fig. 4, B and C).

As the above data demonstrated that both PPAR γ and RXR agonists are effective in the treatment of colitis, we next assessed whether the combination of LG101305 with rosiglitazone could have synergistic beneficial effects on the

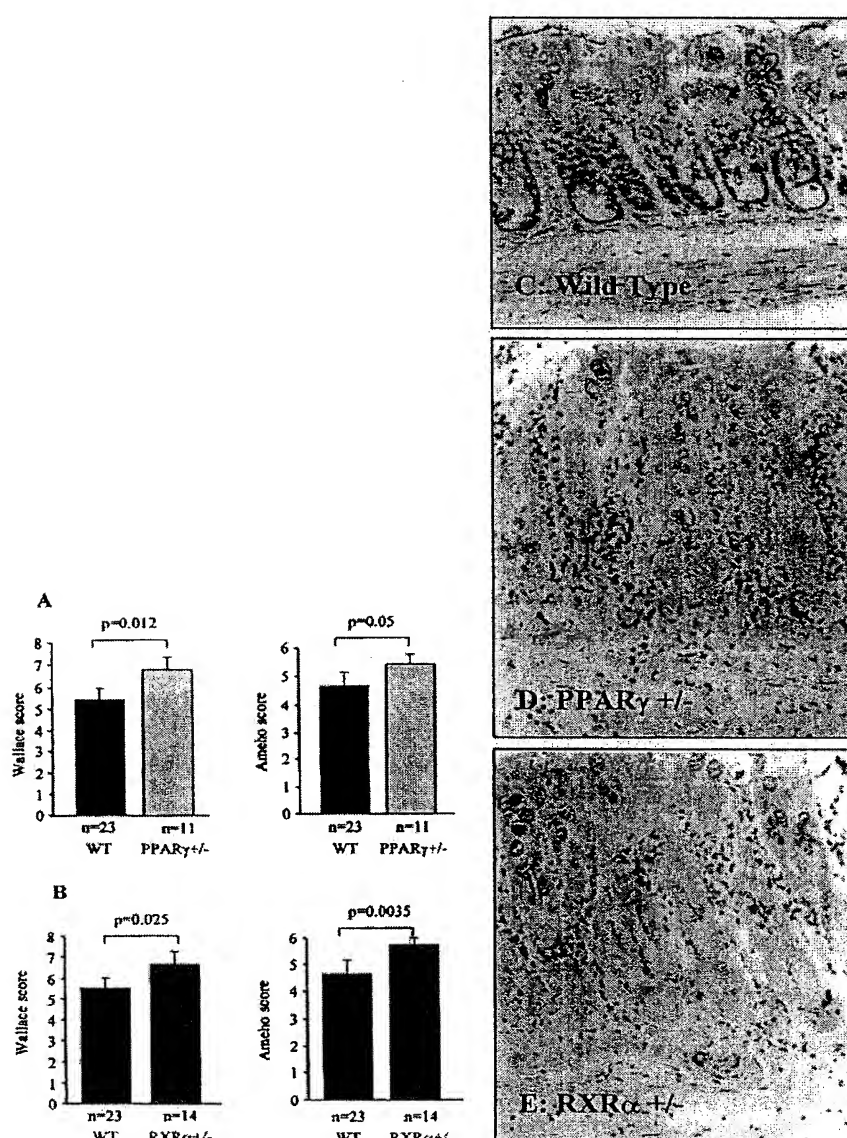
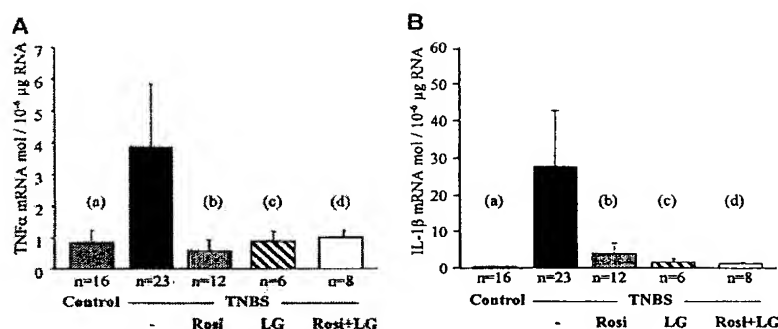


Figure 6. PPAR γ ^{+/-} and RXR α ^{+/-} mice are more susceptible to TNBS-induced colitis. (A and B) Wallace macroscopic and Amecho histologic inflammation scores of 129/Sv wild-type (WT), PPAR γ ^{+/-} (A) and RXR α ^{+/-} (B) mice 2 d after induction of colitis by TNBS administration. (C) Representative transperietal colon section of 129/Sv wild-type mice (Amecho score 5) 2 d after the induction of colitis by TNBS showing a moderate infiltrate with a necrosis limited to the superficial part of the mucosa ($\times 200$). (D and E) Transperietal colon sections in PPAR γ ^{+/-} (D) and RXR α ^{+/-} (E) mice (both Amecho scores of 6) 2 d after the induction of colitis by TNBS. Thickening of the colon wall with a marked transperietal inflammatory infiltrate and necrosis ($\times 200$). Mean \pm SEM are indicated, the number of mice, as well as the statistical significance are indicated.



vs. TNBS; (b) $P = 0.001$ in untreated TNBS colitis vs. mice receiving rosiglitazone; (c) $P = 0.076$ in untreated TNBS colitis vs. mice receiving LG101305; and (d) $P = 0.05$ in untreated TNBS colitis vs. mice receiving both rosiglitazone and LG101305.

TNBS-induced colitis. When both these agonists were given preventively at doses of 1–20 mg/kg/d, striking macroscopic and histologic improvements of TNBS-induced colitis were observed (Fig. 5 B), suggesting a synergistic effects of these two agonists. In fact, lesions were often almost absent and submucosal neutrophil infiltrate was modest, with very low MPO activity (Fig. 4 C). The synergism was best illustrated by the efficacy of the combination of PPAR γ and RXR ligands at a dose of 1 mg/kg/d each, which is 1/20th of the maximally efficacious dose of each individual agonist, and which still retained a remarkable anti-inflammatory effect (Figs. 4 and 5 B).

Increased Susceptibility of PPAR $\gamma^{+/-}$ and RXR $\alpha^{+/-}$ Mice to TNBS-induced Colitis. As the above intervention studies using PPAR γ and RXR agonists were suggestive of the involvement of the RXR/PPAR γ heterodimer in improving colitis, we next tested whether mice heterozygous for a deficiency of PPAR γ and/or RXR α were more susceptible to the development of TNBS-colitis. These heterozygous knockout mice and their wild-type littermates are on a 129/Sv background. Relative to Balb/c mice, TNBS induced a less severe colitis in 129/Sv mice (compare abso-

lute macroscopic and histologic lesion scores in Figs. 4 and 6; $P < 0.001$). Relative to PPAR $\gamma^{+/+}$ littermates, PPAR $\gamma^{+/-}$ mice had more pronounced macroscopic and microscopic lesions with an enhancement of MPO levels (data not shown) after a challenge with TNBS (Fig. 6). These lesions were characterized by large and deep ulcerations with a necrosis and inflammatory infiltrate extending deeply into the muscular layer (Fig. 6 D). Relative to the RXR $\alpha^{+/+}$ mice, the RXR $\alpha^{+/-}$ mice developed also significantly more intense macroscopic and histologic lesions after TNBS administration (Fig. 6, B and E). Like for the PPAR $\gamma^{+/-}$ mice, this increased susceptibility to lesion formation was associated with a dramatic induction of MPO levels (data not shown) in the colon and a necrosis which involved ~90% of the specimen (Fig. 6, B and E). In combination with the data obtained using PPAR γ and RXR agonists (Figs. 2–5), these studies in PPAR γ and RXR $\alpha^{+/-}$ mice implicate the RXR/PPAR γ heterodimer in the protection against intestinal inflammation.

PPAR γ and RXR Agonists Inhibit Inflammatory Cytokine Expression and NF- κ B and JNK/p38 MAPK Activities. Although all the above data underscore the importance of the

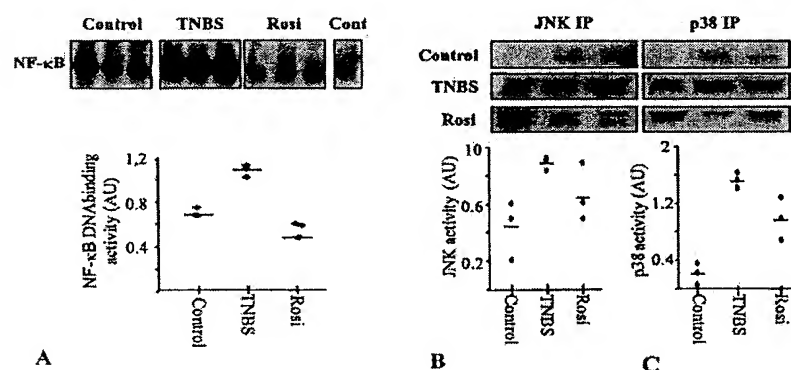


Figure 8. NF- κ B, JNK, and p38 kinase activities 2 d after TNBS colitis induction in mice treated preventively with rosiglitazone. (A) NF- κ B. Proteins in colon homogenates obtained from three control mice (Control), three mice receiving TNBS (TNBS), and three mice receiving TNBS and rosiglitazone (Rosi, 20 mg/kg/day) were used in electrophoretic mobility shift assay (EMSA) reactions as described in Materials and Methods. The top panel shows autoradiographs of the EMSA assay using a radiolabeled NF- κ B consensus binding site. The bottom panel shows a graphical presentation obtained after scanning the corresponding autoradiographs. Compared with the most intense NF- κ B complex radiolabeled signal obtained in mice receiving TNBS, control (Cont) corresponding to a competition with an excess of cold NF- κ B showed a marked decrease of the radiolabeling. (B and C)

JNK (B) and p38 kinase (C) activities in immunoprecipitates of colon extracts obtained from three control mice (Control), three mice receiving TNBS (TNBS), and three mice receiving TNBS and treated preventively with rosiglitazone (Rosi, 20 mg/kg/day). The top panel shows autoradiographs, showing the phosphorylation status of the GST-ATF2 fusion protein used as a substrate to measure the kinase activities in the JNK and p38 immunoprecipitates. The bottom panel shows a graphical presentation obtained after scanning the corresponding autoradiographs. AU, arbitrary units.

RXR/PPAR γ heterodimer in colon inflammation, they did not address any of the possible downstream mechanisms involved in this protective effect. Intestinal inflammatory cytokines and the NF- κ B and MAPK pathways were evaluated in mice killed 2 d after TNBS administration. Low concentrations of TNF- α and IL-1 β mRNA were present in the colon of control mice (Fig. 7). 2 d after induction of colitis by TNBS, TNF- α and IL-1 β mRNA were significantly induced, compatible with a major inflammatory reaction (Fig. 7). In contrast, the preventive administration of rosiglitazone (at 20 mg/kg/d), troglitazone (at 150 mg/kg/d), LG101305 (at 20 mg/kg/d), and the simultaneous administration of both rosiglitazone and LG101305 (both at the dose of 1 mg/kg/d) normalized TNF- α and IL-1 β mRNA concentrations in the colon (Fig. 7 and Table II). Conversely, the more intense macroscopic and histologic colitis observed in PPAR $\gamma^{+/-}$ and RXR $\alpha^{+/-}$ mice were associated with a significant increase in the levels of TNF- α and IL-1 β mRNA, compared with wild-type mice with colitis (data not shown).

Furthermore, in control mice killed 2 d after administration of 50% ethanol or a saline solution, low levels of nuclear NF- κ B DNA binding activity, as well as of JNK and p38 activities, were observed in colon protein extracts (Fig. 8). 2 d after TNBS administration, NF- κ B DNA binding, JNK, and p38 kinase activities were strongly induced (Fig. 8). Preventive administration of rosiglitazone was associated both with an important decrease of the activity of NF- κ B DNA binding, JNK, and p38 activities, suggesting the involvement of MAPKs in TNBS-induced intestinal inflammation in mice (Fig. 8).

Discussion

Intrarectal administration of TNBS to mice provokes a severe colitis, which represents a well-validated model that has many macroscopic and histologic similarities to IBD in human. These similarities include the presence of granulomas, mucosal infiltration of neutrophils, mediated at least in part by TNF- α and IL-1 β overexpression, and activation of the NF- κ B pathway (22–25). Earlier studies have shown that TNBS-induced colitis responds favorably to many of the current therapies for IBD such as sulfasalazine or 5-aminosalicylic acid (26), glucocorticoids (27), cyclosporin (28), and anti-TNF- α antibodies (23). In view of the high level of expression of PPAR γ in the colon (16, 29, 30) and the reported antiinflammatory effects of activation of PPAR γ (5, 6, 31), we have analyzed the contribution of RXR/PPAR γ heterodimers on intestinal inflammatory responses. As the RXR/PPAR γ heterodimer is “permissive” and can be activated both by PPAR γ or RXR ligands, we first analyzed whether administration of specific synthetic PPAR γ or RXR agonists attenuated TNBS-induced colitis. Consistent with the work of Su et al. (7), administration of PPAR γ agonists, such as rosiglitazone or troglitazone to TNBS-treated animals, attenuated the inflammatory response. These results confirm that PPAR γ ligands have antiinflammatory effects in the intestine. Recently, two stud-

ies have shown unequivocally that PPAR γ expression by macrophages is not required for PPAR γ ligands to exert their antiinflammatory effects (32, 33). This absence of direct effects of PPAR γ has only been shown on macrophage biology and not in other cells involved in the inflammatory reaction. Therefore, these observations suggest that the ability of thiazolidinediones to suppress TNBS-induced colitis which involved numerous cells such as macrophages but also lymphocytes, neutrophils, mast cells, eosinophils, and epithelial cells may be mediated at least in part through a different target. The rexinoid LG101305 also had potent antiinflammatory effects in the intestine. Furthermore, simultaneous administration of both PPAR γ and RXR ligands had a markedly synergistic beneficial effect on colitis, enabling a significant dose reduction for each agonist. Further evidence in support of the implication of the RXR/PPAR γ heterodimer in the protection against colon inflammation came from the characterization of PPAR $\gamma^{+/-}$ and RXR $\alpha^{+/-}$ mice, both of which were more susceptible to TNBS-induced inflammation. Taken together, our studies, with synthetic RXR and PPAR γ agonists and with heterozygous deficient mice, show that activators of the RXR/PPAR γ heterodimer might exert a direct and indirect control of inflammatory responses in the intestine. Furthermore, these data suggest that the synergistic antiinflammatory effect of RXR and PPAR γ agonists could be beneficial in a clinical setting, as it might avoid adverse events often encountered when these agonists are used in monotherapy at higher doses.

The persistence of inflammation in deeper layers of the colon in some animals treated with PPAR γ and RXR agonists, despite the repair of mucosal lesions, is particularly interesting. The absence of histologic improvement in the deeper layers of the colon could be related at least in part to the preferential expression of PPAR γ in the colonic mucosa (30) and its absence in the other layers of the colon (more particularly the muscular layer). Thus, activators of the RXR/PPAR γ heterodimer might exert antiinflammatory effects in the mucosa which selectively expresses this heterodimer, and indirectly protects the deeper colon layers against damage by the preservation of the integrity of the mucosal barrier. Further confirmation of this possibility would require the study of animals in which activity of the RXR/PPAR γ heterodimer was specifically eliminated in the intestinal mucosa.

The precise mechanisms by which activation of the RXR/PPAR γ heterodimer negatively regulates intestinal inflammation remains to be elucidated (31), but our data suggest an involvement of both the NF- κ B and stress kinase pathways. Many studies have suggested an important pathogenic role for TNF- α and IL-1 β in the pathophysiology of TNBS-induced colitis through the recruitment of polymorphonuclear cells in the colon (34, 35). This possibility is supported by the overexpression of these cytokines in TNBS-induced colitis, the improvement in disease severity after the neutralization of TNF- α and IL-1 β in vivo, the absence of chronic TNBS-induced colitis in TNF- α knockout mice, and the development of lethal pan-colitis

upon TNBS administration in TNF- α transgenic mice (23). In vivo, the predominant role of NF- κ B during colonic inflammation has been demonstrated in TNBS-induced colitis and in spontaneous colitis of IL-10-deficient mice (22). It is generally believed that the signal transduction pathways activated in response to TNF- α and IL-1 β initiate NF- κ B activation through the NF- κ B-inducing kinase (NIK) signaling pathway (36, 37) and the activation of two I κ B kinases, IKK-1 and IKK-2, which phosphorylate I κ B, leading to its degradation (38). Nonetheless, several reports also demonstrate an involvement of members of the MAPK pathway such as MAPK kinase (MEKK1) (39–42) and JNK or p38 MAPK in the activation of NF- κ B (8–10). In addition, concomitant activation of the JNK/p38 MAPK and NF- κ B pathways has been observed in several cell types, including macrophages (43–46). Despite this suggestive role of stress kinase in NF- κ B activation, the involvement of the MAPK pathway in chronic intestinal inflammation has been neglected. In this study, we demonstrated that NF- κ B, JNK, and p38 are activated concomitantly during TNBS-induced intestinal inflammation in mice and that the activation of both pathways can be attenuated by activation of RXR/PPAR γ heterodimers. Considering the ambiguous relationships between NF- κ B activation and the MAPK signaling pathways (47–50), our observations suggest that activation of both signaling pathways can lead to colon inflammation.

In conclusion, the data obtained with TNBS-induced colitis in mice, an animal model having similarities with IBD in humans, support the existence of an important anti-inflammatory action of RXR/PPAR γ heterodimers in the intestine. Our data further suggest that the simultaneous administration of synthetic PPAR γ and RXR agonists could represent an attractive therapeutic strategy for the treatment of IBD. In addition, they question the possible impact of changing dietary habits on the increased prevalence of IBD and suggest that beneficial effects could be expected from changes in diet.

We thank the various members of the Auwerx lab for discussions and suggestions, as well as C. Bourdon, C. Haby, V. Prima, V. Jérónimo, and R. Paris for their valuable technical assistance.

We enjoyed the support of grants from Institut de Recherche des Maladies de l'Appareil Digestif, the association F. Aupetit, the Centre Hospitalier et Universitaire de Lille (EA2687, CA14555, and PHRC1926), Hôpitaux de Strasbourg, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, l'Association pour la Recherche contre le Cancer (ARC), the Juvenile Diabetes Foundation, the Human Frontiers Science Program, and from Ligand Pharmaceuticals.

Submitted: 18 December 2000

Revised: 20 February 2001

Accepted: 23 February 2001

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